PCT/AU99/01137

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Twenty-fifth day of February 2000

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THE VICTOR CHANG CARDIAC RESEARCH INSTITUTE

PROVISIONAL SPECIFICATION

Invention Title:

Treatment of heart disease

The invention is described in the following statement:

Technical Field

This invention relates to polypeptides which affect organisation of cardiac muscle contractile units, assay for identifying such polypeptides, and methods for improving cardiac function by the administration of such polypeptides to patients with heart disease.

Background of the Invention

Heart failure affects 1.5% of populations, approximately three million Americans, developing at a rate of approximately 400,000 new cases per year in USA. Current therapy for heart failure is primarily directed to using angiotensin-converting enzyme (ACE) inhibitors and diuretics. ACE inhibitors appear to slow progress to end-stage heart failure; however, they are unable to relieve symptoms in more than 60% of heart failure patients and reduce mortality of heart failure only by approximately 15-20%. Heart transplantation is limited by the availability of donor hearts. With the exception of digoxin, the chronic administration of positive inotropic agents has not resulted in a useful drug without adverse side effects, including increased arrhythmias, or sudden death. These deficiencies in our current therapy suggest the need for additional therapeutic approaches.

Growth of cardiac muscle cells switches from proliferation to hypertrophy during heart development. The former process is characterised by an increase in cardiac muscle cell number, and the latter by an increase in cell size without DNA synthesis or cell division. This switch is associated with terminal differentiation of cardiac muscle cells and occurs gradually during heart development, starting during the late embryonic stages and ending a few weeks after birth. During this period, gene expression, particularly that involving the cell cycle and signalling, is reprogrammed. For example, expression of a number of receptor protein tyrosine kinases and other cell cycle components decreases. Cell phenotype is also changed as cell-cell adhesions and contractile proteins are more organised in terminal differentiated myocardial cells.

Adult heart hypertrophy is an important adaptive physiological response to increased demands for cardiac work or after a variety of pathological stimuli that lead to cardiac injury. Normal hypertrophic cells have a large size with increased and well organised contractile units, as well as strong cell-cell adhesions. Although pathologically hypertrophic cells also have large size and accumulation of proteins, they often display

disorganisation of contractile proteins (disarray of sarcomeric structures) and poor cell-cell adhesions (disarray of myofibers). Thus, in pathological hypertrophy, the increase in size and accumulation of contractile proteins are associated with disorganised assembly of sarcomeric structures and a lack of robust cell-cell interactions (Braunwald (1994) in Pathphysiology of Heart Failure, (Braunwald, ed.); Saunsers, Philadelphia; Vol. 14, pp 393-402).

The disarray of myofibers and sarcomeres are important features of cardiomyopathy. The former is a disorder of cell-cell association, and the latter is disorganisation of heart muscle contractile proteins. They are influenced by specific cell signals. Thus, a number of signals, like growth factors and hormones, alter cell adhesion and sarcomeric structure. Without these stimuli, cardiomyocytes display disarray of the cytoskeleton and sarcomeric structures, as well as disassociation of cell-cell interactions. As cardiac muscle cell differentiation is tightly associated with cardiac cell remodelling, adhesion and contractile protein organisations, factors that stimulate myocardial cell differentiation may be critical for enhancing the assembly of adult cardiac muscle cell sarcomeric structures.

Studies in an *in vitro* model system of cardiac muscle cell have led to the identification of a number of mechanical, hormonal, growth factor, and pathological stimuli which can activate several independent phenotype features of cardiac hypertrophy (Chien et al. (1991) FASEB J. 5:3037-3046; Zhou et al., (1995) PNAS. USA, 92:7391-7395). Currently, there are at least three signal transduction pathways, involving both *ras*-, *rho*- and G_q protein-dependent downstream effectors implicated in the activation of features of the hypertrophic response in these *in vitro* model systems. While a great deal of progress has been made in uncovering the signalling pathways which activate the ventricular muscle cell hypertrophic response, relatively little is known about the mechanisms which specifically stimulate terminal differentiation of cardiac muscle cells and the terminal differentiation-associated assembly of contractile proteins. Compounds that could influence these processes may be form a major new class of therapeutics for the treatment of a variety of cardiac diseases.

Neuregulins, a family of EGF-like growth factors, activate erbB receptor tyrosine kinases that belong to the EGF receptor superfamily, and are involved in an array of biological responses: stimulation of breast cancer cell differentiation and secretion of milk proteins; induction of neural crest cell

differentiation to Schwann cells; stimulation of skeletal muscle cell synthesis of acetylcholine receptors; and, promotion of myocardial cell survival and DNA synthesis. *In vivo* studies of neuregulin gene-targeted homozygous mouse embryos with severe defects in ventricular trabeculae formation and dorsal root ganglia development indicate that neuregulin is essential for heart and neural development. However, information on how neuregulin controls cell differentiation and its downstream signalling pathways is limited.

Within the heart, neuregulin and ErbB receptors are respectively expressed in the endocardial lining and cardiac muscle layer in early stages of development. Since these two layers are widely separated, the neuregulin ligand must transverse the space between the two cell layers to activate their cognate ErbB receptors. Activation of these receptors in myocardial cells is necessary for promoting muscle cell growth or migration toward the endocardium, which results in the formation of finger-like structures (ventricular trabeculae) between these two layers. It is not clear previously if neuregulin stimulates myocardial cell differentiation.

The present inventors have now found that neuregulin and its cellular actions may be suitable for use in detection, diagnosis and treatment of heart disease.

Summary of the Invention

The present invention is based in part on the discovery that neuregulin enhances cardiac muscle cell differentiation and organisation of sarcomeric and cytoskeleton structures, as well as cell-cell adhesion. Neuregulin, neuregulin polypeptide, neuregulin derivatives, or compounds which mimic the activities of neuregulins, fall within the scope of the methods of the present invention and are abbreviated hereinafter as NRG.

In a first aspect, the present invention consists in a method of causing cardiomyocyte growth and/or differentiation, the method comprising exposing the cardiomyocyte to NRG thereby activating the MAP kinase pathway in the cardiomyocyte and causing growth and/or differentiation of the cardiomyocyte.

In a second aspect, the present invention consists in a method of inducing remodelling of muscle cell sarcomeric and cytoskeleton structures, or cell-cell adhesions, the method comprising treating the cells with neuregulin thereby activating the MAP kinase pathway in the cells and causing remodelling of the cell structures or the cell-cell adhesions.

It will be appreciated that neuregulin may be provided directly to the cell or provided indirectly by causing neuregulin to be produced in cells by inducing expression of the gene(s) involved in neuregulin production. The production may be in the same cell to which the method is directed in an autocrine manner or by some other cell in a paracrine manner.

In a third aspect, the present invention consists in a method of identifying polypeptides or compounds which stimulate cardiac muscle cell differentiation, the method comprising contacting the cardiac muscle with a test polypeptide or compound in the presence of an inducer of cardiac muscle cell proliferation in the form of neuregulin, and measuring the development of cardiac muscle cell differentiation.

The differentiation of cardiac muscle cells is measured in cells exposed to neuregulin or other test polypeptides, or to a mixture of neuregulin with a test polypeptide. Differentiation of cardiac muscle cell is measured in a variety of ways, including by calculation of increases or decreases in DNA synthesis, analysis of the time-course of phosphorylation of MAP kinases in cardiac muscle cells, evaluation of the expression of cell cycle inhibitor, $p21^{CIP1}$, phenotypic organisation of contractile units, accumulation of contractile units, phenotypic alteration of cytoskeleton actin fibers, and the phenotype of cell-cell adhesions.

In a first embodiment of the method of identifying polypeptides or compounds which stimulate cardiac muscle cell differentiation, cells are incubated with different concentrations of various peptides or compounds and the effect of the test peptide or compound in different concentrations on cardiac muscle cell differentiation measured.

In a second embodiment of identifying polypeptides or compounds which induce cardiac muscle cell differentiation that dominates over that of the putative inducer of cardiac muscle cell proliferation, insulin-like growth factor-1 (IGF-1), cells are incubated with IGF-1, with and without the test polypeptide or compound, and the ability of the test polypeptide or compound to inhibit IGF-1-mediated cardiac muscle cell DNA synthesis, assembly of sarcomeric structures and cell-cell adhesions are measured.

In a third embodiment, the cells are incubated with phenylephrine (PE) with and without the test polypeptide or compound, and the ability of the test polypeptide or compound to augment PE-mediated cardiac muscle cell differentiation is determined. A test polypeptide which stimulates

cardiac muscle cell differentiation may stimulate the assembly of sarcomeres and thus enhance heart function in a variety of ways, including by activating neuregulin-specific receptors, e.g., ErbB2, ERbB3 and ErbB4.

In a fourth aspect, the present invention consists in a method of identifying polypeptides or compounds which inhibit neuregulin stimulation of ventricular muscle cell differentiation, the method comprising contacting the ventricular muscle cell with the test polypeptide or compound in the presence neuregulin and measuring any inhibition of neuregulin stimulation of the ventricular muscle cell.

A compound may inhibit neuregulin stimulation of ventricular muscle cell differentiation by blocking, suppressing, reversing, or antagonising the action of neuregulin. In one embodiment, the measurement is by detecting DNA synthesis of ventricular muscle cells.

In a fifth aspect, the present invention consists in a therapeutic method of treating or preventing disassociation of cardiac muscle cell-cell adhesion and/or the disarray of sarcomeric structures in a mammal, the method comprising administering to the mammal a therapeutically effective amount of a neuregulin or its derivatives.

In one embodiment, the therapeutic method is directed to treating heart failure resulting from disassociation of cardiac muscle cell-cell adhesion and/or the disarray of sarcomeric structures in the mammal.

In a sixth aspect, the present invention consists in a method of preventing or lowering the incidence of heart disease in a mammal, the method comprising preventing or lowering the interference or effects of polypeptides or compounds on the action of neuregulin and its receptors, ErbBs, that produces heart failure.

In another embodiment, a therapeutic agent which mimics the effects of neuregulin is used to treat or prevent PE, or IGF-1-mediated cardiac muscle cell dysfunction.

In an seventh aspect, the present invention consists in a method of determining predisposition to heart disease or heart failure in a subject, the method comprising testing cardiac or related cells of the subject for the ability to express and/or produce normal or adequate levels of neuregulin or its cognate ErbB receptors. The inability to express and/or produce normal or adequate levels of neuregulin being indicative of predisposition to heart disease or heart failure.

In a eighth aspect, the present invention consists in the use of neuregulin, neuregulin polypeptide, neuregulin derivatives, or compounds which mimic the activities of neuregulins in the treatment or management of heart disease and heart failure.

By using primary cultured myocardial cells as a model system, the present inventors evaluated neuregulin signalling in cardiac myocyte differentiation, maturation and assembly or maintenance of sarcomeric and cytoskeleton structures. To assay the neuregulin effect on cell signalling, embryonic cardiac muscle cells were incubated with recombinantly purified human neuregulin ligand (rhNRGβ2). Neuregulin at 10^{-8}M resulted in sustained activation of MAP kinases for at least 21 hours, whereas only transient activation was observed with a lower concentration (10^{-10}M) of rhNRGβ2. Expression of the Cdk inhibitor, $p21^{CIP1}$, was enhanced by the 10^{-8}M, but not the 10^{-10}M concentration of the ligand. The higher ligand concentration, concomitant with this increase in $p21^{CIP1}$ expression, resulted in a decrease in DNA synthesis, that was associated with terminal differentiation, whereas an increase in DNA synthesis and continued proliferation was observed with the lower dose. Furthermore, when neuregulin was mixed with IGF-1, rhNRGβ2 at either concentrations (10^{-8}M, or 10^{-10}M) did not show a negative effect on the DNA synthesis and significantly blocked IGF-I-stimulated cardiomyocyte proliferation. To further evaluate the NRG-stimulated myocardial cell differentiation, sarcomeric and cytoskeleton structures of cultured neonatal rat cardiac muscle cells were examined by Phalloidin staining and immunofluorescent staining with anti-actinin antibody. rhNRGβ2 dramatically improved sarcomeric and cytoskeleton structures, as well as cell-cell adhesions. Such an effect was not found from the cells stimulated with either insulin IGF-1 or PE. When rhNRGβ2 was mixed with either IGF-1 or PE, rhNRGβ2 improved the cell structures. The 10^{-8}M concentration of rhNRGβ2 showed maximal effect on improvements of sarcomeres and cell-cell adhesions. In addition, neuregulin overrode the negative regulation of MHC-α expression mediated by PE stimulation. These findings indicate that NRG function through two distinct pathways: one activated at lower ligand concentrations results in cardiomyocyte growth, whereas the other, activated with higher concentrations, is mediated by sustained activation of the MAP kinase pathway and results in terminal differentiation and maturation.

Throughout this specification, unless the context requires otherwise, the word "comprise", or variations such as "comprises" or "comprising", will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

In order that the present invention may be more clearly understood, preferred forms will be described with reference to the following examples and drawings.

Brief Description of the Drawings

Figure 1. Effects of neuregulin on calls. Neuregulin decreases embryonic cardiac muscle cell DNA synthesis in a concentration dependent manner. Purified cardiac myocytes were plated onto gelatin-coated 96-well plates with addition of a vehicle control (open square), recombinant human NRGβ2 (rhNRGβ2) (close triangle) or insulin-like growth factor-I (IGF-I) (open circle) at the concentrations indicated. DNA synthesis was measured using [^{3}H]thymidine incorporation after 24 hr incubation with the ligands. Data are shown as the mean value of five wells with standard deviation.

Figure 2. Neuregulin stimulation results in a sustained activation of MAP kinases in cardiac muscle cells. **(a)** Cells cultured in serum-free medium were stimulated with the vehicle alone (-) or rhNRGβ2 at either 10^{-10}M or 10^{-8}M for certain periods as indicated. The treated cells were harvested and subjected to a Western blot using specific phospho-MAPK antibody. Normalisation was conducted by use of Western blotting with anti-erbB2 antibody. **(b)** The band intensities were quantified by densitometry and displayed as two time course curves: responses of MAP kinase to the 10^{-8}M ligand (close triangle) and 10^{-10}M ligand (open circle).

Figure 3. Neuregulin stimulation results in an enhanced expression of $p21^{CIP1}$ in cardiac muscle cells. **(a)** Cultured cardiac muscle cells were stimulated either with 10^{-10}M or 10^{-8}M of rhNRGβ2 in the absence or presence of serum (5% of FBS) for 24 hr. Cells were lysed in electrophoresis sample loading buffer and subjected to Western blot analysis. The $p21^{CIP1}$ was detected by a monoclonal anti-$p21^{CIP1}$ antibody. The amount of protein loaded was normalised by detection of erbB2 expression. **(b)** Results of densitometry analysis of bands on the first row of panel a are displayed as expression of $p21^{CIP1}$ without ligand stimulation (clear or horizontal bars),

with the lower concentration ligand stimulation (grey or vertical bars), or with the higher concentration ligand (black or hatched bars).

Figure 4. Neuregulin inhibits IGF-1 stimulated DNA synthesis in cardiac muscle cells. Cells cultured in 96-well plates were stimulated with the optimal concentration of IGF-I (10^{-9}M) in the absence or presence of rhNRGβ2 at concentrations of either 10^{-10}M or 10^{-8}M. DNA synthesis was measured using [^{3}H]thymidine incorporation after 24 hr incubation. Bars are shown as the mean value of data from five wells with standard deviation. Results were from at least thrice repeating the experiments.

Figure 5. Neuregulin enhances actin fiber organisation in cardiac muscle cells. Neonatal rat cardiac muscle cells cultured in serum free medium with (a) or without (b) rhNRGβ2 at concentration 10^{-8}M were fixed and stained with Phalloidin. Cardiac muscle actins were revealed as red fibers in cells with the UV fluorescent microscopy. Photos were taken with a 40X object.

Figure 6. Neuregulin enhances the assembly and organisation of sarcomere proteins, and cell-cell adhesions. Neonatal rat cardiac muscle cells were cultured in serum free medium **(a)**, supplemented with 10^{-10}M **(b)**, or 10^{-8}M (c) rhNRGβ2 for 48 hr. Cells then were immuno-fluorescently stained with anti-α-actinin antibody. Cardiac actinins in sarcomeres were visualised as green striated fibers in the UV fluorescent microscopy. Photos were taken with a 40X object.

Figure 7. Neuregulin improves the sarcomeric structures and cell-cell adhesions in cardiac muscle cells stimulated by IGF-1. Cardiac muscle cells were cultured with in serum free medium supplemented with 10^{-9}M IGF-1 **(a)**, or 10^{-9}M IGF-1 and 10^{-8}M rhNRGβ2 **(b)** for 48 hr. The cells were then fixed and immuno-fluorescently stained with anti-α-actinin antibody. Cardiac actinins in sarcomeres were visualised as green striated fibers in the UV fluorescent microscopy. Photos were taken with a 40X object.

Figure 8. Neuregulin improves the sarcomeric structures and cell-cell adhesions in cardiac muscle cells stimulated by PE. Cardiac muscle cells were cultured with in serum free medium supplemented with 10^{-4}M PE **(a)**, or 10^{-4}M PE and 10^{-8}M rhNRGβ2 **(b)** for 48 hr. The cells were then fixed and immuno-fluorescently stained with anti-α-actinin antibody. Cardiac actinins in sarcomeres were visualised as green striated fibers in the UV fluorescent

microscopy. Photos were taken with a 40X object. Two cell phenotypes were observed with PE alone stimulation and labelled by as A and B.

Figure 9. Neuregulin induces PE-suppressed myosin heavy chain α isoform (MHC-α) expression. Myocardial cells were cultured in serum free medium and separately supplemented with PE, NRG, or PE and NRG as indicated. Relative expression levels of MHC-a and MHC-b were measured by RT-PCR with a lower cycle number (20 cycles). The amount of PCR products represents relative expression levels of these two genes.

Modes for Carrying Out the Invention

Utilising an *in vitro* system of cardiac muscle cell differentiation, a role for neuregulin in stimulating the activation of the differentiation response in comparison with two well-defined hormonal and growth factor stimuli, α_1-adrenergic agonists and IGF-1 has been demonstrated. The present inventors have demonstrated that neuregulin differentiation pathways exist within cardiac muscle cells, and that neuregulin polypeptides can activate these pathways. Since cardiac muscle cell differentiation includes the processes of organisation of sarcomeric structures and cell-cell adhesions, the invention, thus, provides a useful method for the treatment and prevention of cardiac muscle cell with disorganisation of the sarcomeric structures and cell-cell adhesions, and the enhancement of heart function in cardiomyopathy, and for identifying polypeptides or compounds which activate cardiac muscle differentiation pathways.

Before the methods of the invention are described, it is to be understood that this invention is not limited to the particular methods described. The terminology used herein is for the purpose of describing particular embodiments only.

As used in this specification, the singular forms "a", "an", and "the" include plural references unless the context clearly dictates otherwise. Thus, for example, references to "neuregulin" or "a neuregulin peptide" includes mixtures of such neuregulins, neuregulin isoforms, and/or neuregulin-like polypeptides. Reference to "the formulation" or "the method" includes one or more formulations, methods, and/or steps of the type described herein and/or which will become apparent to those persons skilled in the art upon reading this disclosure.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary

skill in the art to which the invention belongs. Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference for the purpose of disclosing and describing material for which the reference was cited in connection with.

Definitions

"Neuregulin or neuregulin analogs" are molecules that can activate ErbB2/ErbB4 or ErbB2/ErbB3 heterodimer protein tyrosine kinases, such as all neuregulin isoforms, neuregulin EGF domain alone, neuregulin mutants, and any kind of neuregulin-like gene products that also activate the above receptors. The "neuregulin" used in this invention is the following polypeptide which is a fragment of human neuregulin β2 isoform containing the EGF-like domain, the receptor binding domain.

The cDNA sequence:

AGCCATCTTGTAAATGTGCGGAGAAGGAGAAAACTTTCTGTGTGAATGG
AGGGGAGTGCTTCATGGTGAAAGACCTTTCAAACCCCTCGAGATACTTGT
GAGGAGCTGTACCAG

The amino acid sequence encoded by the above DNA sequence:

SHLVKCAEKTFCVNGGECFMVKDLSNPSRYLCKCPNEFTGDRCQNYVMASF
YKAEELY

"Cardiac muscle cell differentiation" is a condition characterised by the decrease in DNA synthesis by more than 10%, inhibition of other factor-stimulated DNA synthesis more than 10%, well organised sarcomeric structures and cell-cell adhesions, sustained activation of MAP kinases, and enhanced expression of $p21^{CIP1}$.

"Organised, or enhanced organisation of sarcomeres or sarcomeric structures" is a condition characterised by the straight array of contractile proteins revealed by immunofluorescent staining of α-actinin in cardiac muscle cells. The straight array of α-actinin proteins in cells can be distinguished by microscopy and its connected photography as exampled in Figures of this specification.

"Disorganised or disarray of sarcomeres or sarcomeric structures" is the opposite meaning of the above definitions.

"Organised, or enhanced organisation of cytoskeleton structures" is a condition characterised by the straight actin fibers revealed by phalloidin

staining of cardiac muscle cells. The straight actin fibers in cells can be distinguished by microscopy and its connected photography as exampled in Figures of this specification.

"Disorganised or disarray of cytoskeleton structures" is the opposite meaning of the above definitions.

"Sustained activation of MAP kinases" is that phosphorylated state of MAP kinases, p42/44, is maintained for at least 21 hr in cells.

"Enhanced expression of $p21^{CIP1}$" is that expression of $p21^{CIP1}$ is increased at least 50% that is maintained for at least 24 hr in cells.

"The treatment of heart diseases" includes all suitable kinds of methods, such as vein injection of the neuregulin polypeptide, and gene therapy methods, in which heart or other cells were forced to contain a gene encoding neuregulin or derivatives for the treatment of heart diseases. For example, Adenovirus or Adeno-Associated-Virus can be used as a carrier to deliver neuregulin gene into infected heart or other cells. The infected cell can then express and secret neuregulin polypeptide to activate ErbBs on cardiac muscle cells.

"Ventricular muscle cell hypertrophy" is a condition characterised by an increase in the size of individual ventricular muscle cells, the increase in cell size being sufficient to result in a clinical diagnosis of the patient or sufficient as to allow the cells to be determined as larger (e.g., 2-fold or more larger than non-hypertrophic cells). It may be accompanied by accumulation of contractile proteins within the individual cardiac cells and activation of embryonic gene expression.

In vitro and *in vivo* methods for determining the presence of ventricular muscle cell hypertrophy are known. *In vitro* assays for ventricular muscle cell hypertrophy include those methods described herein, e.g., increased cell size and increased expression of atrial natriuretic factor (AND). Changes in cell size are used in a scoring system to determine the extent of hypertrophy. These changes can be viewed with an inverted phase microscope, and the degree of hypertrophy scored with an arbitrary scale of 7 to 0, with 7 being fully hypertrophied cells, and 3 being non-stimulated cells. The 3 and 7 states may be seen in Simpson et al. (1982) Circulation Res. 51: 787-801, Figure 2, A and B, respectively. The correlation between hypertrophy score and cell surface area (μm^2) has been determined to be linear (correlation coefficient = 0.99). In phenylephrine-induced hypertrophy, non-exposed

(normal) cells have a hypertrophy score of 3 and a surface area/cell of 581μm^2 and fully hypertrophied cells have a hypertrophy score of 7 and a surface area/cell of 1811 μm^2, or approximately 200% of normal. Cells with a hypertrophy score of 4 have a surface area/cell of 771 μm^2, or approximately 30% greater size than non-exposed cells; cells with a hypertrophy score of 5 have a surface area/cell of 1109 μm^2, or approximately 90% greater size than non-exposed cells; and cells with a hypertrophy score of 6 have a surface area/cell of 1366 μm^2, or approximately 135% greater size than non-exposed cells. The presence of ventricular muscle cell hypertrophy preferably includes cells exhibiting an increased size of about 15% (hypertrophy score 3.5) or more. Inducers of hypertrophy vary in their ability to induce a maximal hypertrophic response as scored by the above-described assay. For example, the maximal increase in cell size induced by endothelin is approximately a hypertrophy score of 5.

"Suppression" of ventricular muscle cell hypertrophy means a reduction in one of the parameters indicating hypertrophy relative to the hypertrophuc condition, or a prevention of an increase in one of the parameters indicating hypertrophy relative to the normal condition. For example, suppression of ventricular muscle cell hypertrophy can be measured as a reduction in cell size relative to the hypertrophic condition. Suppression of ventricular muscle cell hypertrophy means a decrease of cell size of 10% or greater relative to that observed in the hypertrophic condition. More preferably, suppression of hypertrophy means a decrease in cell size of 30% or greater; most preferably, suppression of hypertrophy means a decrease of cell size of 50% or more. Relative to the hypertrophy score assay when phenylephrine is used as the inducing agent, these decreases would correlate with hypertrophy scores of about 6.5 or less, 5.0-5.5, and 4.0-5.0, respectively. When a different agent is used as the inducing agent, suppression is measure relative to the maximum cell size (or hypertrophic score) measured in the presence of that inducer.

Prevention of ventricular muscle cell hypertrophy is determined by preventing an increase in cell size relative to normal cells, in the presence of a concentration of inducer sufficient to fully induce hypertrophy. For example, prevention of hypertrophy means a cell size increase less than 200% greater than non-induced cells in the presence of maximally-stimulating concentration of inducer. More preferably, prevention of

hypertrophy means a cell size increase less than 135% greater than non-induced cells; and most preferably, prevention of hypertrophy means a cell size increase less than 90% greater than non-induced cells. Relative to the hypertrophy score assay when phenylephrine is used as the inducing agent, prevention of hypertrophy in the presence of a maximally-stimulating concentration of phenylephrine means a hypertrophic score of about 6.0-6.5, 5.0-5.5, and 4.0-4.5, respectively.

In vivo determination of hypertrophy include measurement of cardiovascular parameters such as blood pressure, heart rate, systemic vascular resistance, contractility, force of heart beat, concentric or dilated hypertrophy, left ventricular systolic pressure, left ventricular mean pressure, left ventricular end-diastolic pressure, cardiac output, stroke index, histological parameters, and ventricular size and wall thickness. Animal models available for determination of development and suppression of ventricular muscle cell hypertrophy *in vivo* include the pressure-overload mouse model, RV murine dysfunctional model, transgenic mouse model, and post-myocardial infarction rat model. Medical methods for assessing the presence, development, and suppression of ventricular muscle cell hypertrophy in human patients are known, and include, for example, measurements of diastolic and systolic parameters, estimates of ventricular mass, and pulmonary vein flows.

The terms "treatment", "treating" and the like are used herein to generally mean obtaining a desired pharmacological and/or physiological effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment" as used herein covers any treatment of a disease in a mammal, particularly a human, and includes:

preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it;

inhibiting the disease, i.e., arresting its development; or

relieving the disease, i.e., causing regression of the disease.

The invention is directed to treating patients with or at risk for development of heart disease and related conditions, e.g., heart failure. More specifically, "treatment" is intended to mean providing a therapeutically detectable and beneficial effect on a patient suffering from heart disease.

By the term "heart failure" is meant an abnormality of cardiac function where the heart does not pump blood at the rate needed for the requirements of metabolising tissues. Heart failure includes a wide range of disease states such as congestive heart failure, myocardial infarction, tachyarrythmia, familial hypertrophic cardiomyopathy, ischaemic heart disease, idiopathic dilated cardiomyopathy, and myocarditis. The heart failure can be caused by any number of factors, including ischaemic, congenital, rheumatic, or idiopathic forms. Chronic cardiac hypertrophy is a significantly diseased state which is a precursor to congestive heart failure and cardiac arrest.

"Treatment" refers to both therapeutic treatment and prophylactic or preventative measures, wherein the object is to prevent or slow down (lessen) hypertrophy. Those in need of treatment include those already with the disorder as well as those prone to have the disorder or those in which the disorder is to be prevented. The hypertrophy may be from any cause which is responsive to retinoic acid, including congenital, viral, idiopathic, cardiotrophic, or myotrophic causes, or as a result of ischaemia or ischaemic insults such as myocardial infarction. Typically, the treatment is performed to stop or slow the progression of hypertrophy, especially after heart damage, such as from ischaemia, has occurred. Preferably, for treatment of myocardial infarctions, the agent(s) is given immediately after the myocardial infarction, to prevent or lessen hypertrophy.

The terms "synergistic, "synergistic effect" and like are used herein to describe improved treatment effects obtained by combining one or more therapeutic agents with one or more retinoic acid compounds. Although a synergistic effect in some fields is meant an effect which is more than additive (e.g., 1+1=3), in the field of medical therapy an additive (1+1=2) or less than additive (1+1=1.6) effect may be synergistic. For example, if each of two drugs were to inhibit the development of ventricular muscle cell hypertrophy by 50% if given individually, it would not be expected that the two drugs would be combined to completely stop the development of ventricular muscle cell hypertrophy. In many instances, due to unacceptable side effects, the two drugs cannot be administered together. In other instances, the drugs counteract each other and slow the development of ventricular muscle cell hypertrophy by less than 50% when administered together. Thus, a synergistic effect is said to be obtained if the two drugs

slow the development of ventricular muscle cell hypertrophy by more than 50% while not causing an unacceptable increase in adverse side effects.

MATERIALS AND METHODS

Reagents and Antibodies

The following antibodies and reagents were used: IGF (*Boehringer*); collagenase (*Worthington*); pancreatin (*Gibco BRL*); MEK1 (MAPKK) inhibitor (PD98059) (*New England*); [methyl-^{3}H]thymidine (*Amersham*); monoclonal anti-erbB2 antibody (*Novocastra*); monoclonal IgG_{2b} $p21^{CIP1}$ (F-5) *(Santa Cruz)*; monoclonal anti-phospho-tyrosine horse radish peroxidase (HRPO)-conjugated antibody, RC20 (*Transduction Laboratories*); monoclonal anti-α-sacromeric actin antibody (clone 5c5), HRPO-conjugated anti-rabbit Ig, and anti-mouse Ig (*Sigma*); PhosphoPlus® p44/42 MAP kinase (Thr202/Tyr204) antibody kit (*New England*); anti-FLAG® M1 affinity gel and anti-FLAG M2 monoclonal antibody (*Eastman Kodak*).

Recombinant human NRGβ2 expression and purification

A cDNA encoding the EGF-like domain of human NRGβ2 isoform (rhNRGβ2), residues 177-237, was inserted into the pFLAG1 expression vector (*IBI*) (a gift from Dr. Rodney J. Fiddes, Co-operative Research Centre for Biopharmaceutical Research, Australia). rhNRGβ2 with a FLAG-peptide attached at its N-terminus, was expressed in the periplasmic space of *E.coli* DH5α, and purified by affinity chromatography using anti-FLAG M1 monoclonal antibody according to the manufacturer's instructions. The purity of rhNRGβ2 was more than 90%.

Primary cultures of mouse cardiac myocytes

Mouse embryos (E11.5-12.5) were used to prepare primary cardiac myocytes. Heart tissue was isolated aseptically from embryos as described previously, and washed with 5 ml of phosphate-buffered saline (PBS, pH7.4) three times. The tissue was digested with 0.05% Trypsin/0.57 mM EDTA at 37^{0}C for 15 min. The dissociated cells from this treatment were discarded, and the remaining tissue was dissociated in a solution of 0.41 mg/ml collagenase and 0.6 mg/ml pancreatin in PBS buffer at 37^{0}C shaker (100 rpm) for 15-30 min. The dissociated cells from the subsequent two treatment were mixed with an equal volume of cold foetal bovine serum (FBS) to stop the digestion reaction, and centrifuged at 1500 rpm for 5 min at room temperature. The supernatant was discarded and the pellet was resuspended in Dulbecco's modified Eagle's medium (DMEM) (*Gibco BRL*) supplemented

with 10% FBS, 10 mM glutamine, 100 units/ml penicillin and streptomycin. The cells were selectively enriched for cardiac myocytes by preplating in growth medium on dishes 3-5 times (1 hour each). The myocytes were plated at a final density of 2.5×10^5 per well on gelatin-precoated six-well plates or 2×10^3 per well on gelatin-precoated 96-well plates at 70-80% confluent for 20 hr. Serum was then withdrawn for 24 hr before cells were treated with NRG or other growth factors in serum-free medium (DMEM) at 37^0C.

Using this method, the present inventors routinely obtained primary cultures with >90% myocytes (data not shown). This was assessed by microscopic observation of cells immuno-fluorescently stained with a monoclonal anti-α-sacromeric actin antibody (clone 5c5) (*Sigma*).

Immunocytochemistry of myocardial cells

After rinsing the cultured cardiac muscle cells with PBS they were fixed with 4% paraformaldehyde plus 0.1% Triton X-100 at room temperature for 30 minutes. The fixed cells were then blocked with 5% skimmed milk in PBS for 30 minutes, followed by incubation with anti-α-actinine antibody (*SIGMA)* for 45 minutes at room temperature. After washing, anti-mouse IgG conjugated with FITC (*Silenus Laboratory, Australia*) incubated with the cells for another half hour. After washing, cells were mounted with 1% *p*-phenylenediamine (1 mg/ml, Sigma) in glycerol, and then covered and sealed. Cells were examined using a UV fluorescence microscope and photographed with a 40x power objective. For Phalloidin staining, cells were fixed in 4% formadehyde for 1 hr at room temperature. After twice washing with PBS, cells were stained by phalloidin for 1 hr in the dark. Cells were then washed 5 times with PBS and mounted with 1% *p*-phenylenediamine (1 mg/ml, Sigma) in glycerol for the UV fluorescent microscopy.

Phosphorylation of $p185^{neu}$ and MAP kinase

Serum-starved cardiac myocytes in 6-well plates were exposed to rhNRGβ2 for various times to activate erbB receptors and MAP kinases. Cells were then immediately lysed in 200 µl of cold lysis buffer (50 mM Hepes, pH 7.5, 150 mM NaCl, 10% Glycerol, 1% (v/v) Triton X-100, 1.5 mM $MgCl_2$, 1 mM ethylene glycol tetraacetic (EGTA), 10 mM sodium pyrophosphate, 10 mM NaF, 2 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 µg/ml aprotinin and 10 µg/ml leupeptin). The resulting lysates were subjected to immunoblotting with HRPO-conjugated monoclonal

antibody RC20 (1:2,000) for detection of erbB receptors and a phospho-specific p44/42 MAP kinase antibody (dilution ratio 1:1,000) for detection of phosphorylated MAP kinases, using an enhanced chemiluminescence (ECL) immunoblotting detection system (*Amersham*). Anti-erbB receptor antibodies were used to normalise for protein loading. Bands on films resulting from fluorescently labelled proteins were quantitated by densitometry.

Expression of $p21^{CIP1}$ protein

Serum-starved cells were stimulated with various concentrations of rhNRGβ2 with or without the MEK inhibitor, PD98059, for 24 hr, lysed in sample loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 0.1% bromophenol blue and 10% glycerol), and subjected to immunoblot analysis with $p21^{CIP1}$ antibody (1:1,000). After probing with HRPO-conjugated anti-mouse Ig (1:3,000), $p21^{CIP1}$ was detected using the ECL immunoblotting detection system. The amount of protein loaded was normalised by probing with antibody to erbB2 receptor after stripping. Immunoblots were quantitated by densitometry.

Thymidine Incorporation

After serum-starvation for 24 hr, the cells were incubated with rhNRGβ2 or IGF-I containing serum-free DMEM for 12 hr, and [*methyl* -^{3}H] thymidine (0.5μ Ci/well) was then added and the cells incubated for a further 12 hr. Cells were rinsed twice with ice-cold 5% trichloroacetic acid and then five times with ice-cold PBS. The cells were dissolved in 100 μl of 1% SDS, and counted in a liquid scintillation counter. All data are expressed as mean ± SEM.

RESULTS

DNA synthesis in primary embryonic mouse cardiomyocytes (E11.5-12.5) was evaluated to investigate their growth response to NRG following stimulation with rhNRGβ2, the recombinantly-produced EGF domain of the human NRGβ2 isoform. As shown in Figure 1, rhNRGβ2 at a concentration of 10^{-10}M produced an approximately 2-fold increase in the DNA synthesis. However, the degree of stimulation of DNA synthesis markedly decreased as ligand concentration increased. By contrast with the response to rhNRGβ2, exposure to recombinant human insulin-like growth factor 1 (IGF-1) caused myocardial cells to show only a proliferative response that was independent of the concentration of growth factor applied to the cells. Inhibition of DNA

synthesis by the higher concentration of NRG was not due to contaminating *E.coli* proteins in the rhNRGβ2 reagent, as proteins purified from bacteria transformed with the FLAG-vector alone, did not have a negative effect on myocardial cell proliferation.

Activation of the erbB receptor family initiates a cascade of molecular interactions, ultimately resulting in the stimulation of MAP kinases. As reported previously, the duration of MAP kinase activation is critical for cell differentiation (Traverse et al. (1994) Current Biology 4:694-701). The time course of MAP kinase activation after either 10^{-8}M or 10^{-10}M rhNRGβ2 treatment, was, therefore, investigated, using a specific-phospho-MAP kinase antibody, which recognises activated p42/p44 MAP kinases. As shown in Figure 2, activation of p42/p44 MAP kinases that was sustained for at least 21 hours was observed with the higher dose of rhNRGβ2, whereas MAP kinase activation was transient at the lower ligand concentration, and fell to the basal level in less than 3 hours.

Since transient activation of MAP kinase is directly related to the temporal expression of $p21^{CIP1}$, and accumulation of $p21^{CIP1}$ leads to cell cycle arrest at the G1 phase, the present inventors questioned whether the sustained activation of MAP kinases leads to a higher level of $p21^{CIP1}$ expression. As shown in Figure 3, an increase in $p21^{CIP1}$ expression was observed only with the higher concentration of rhNRGβ2. This effect on $p21^{CIP1}$ expression was independent of the cell culture conditions used, since similar effects were observed with both serum-free and serum-containing culture media. Enhanced $p21^{CIP1}$ expression with 10^{-8}M rhNRGβ2 was sustained for at least 24 hours, and, thus, is critical for the inhibition of DNA synthesis in NRG-stimulated cardiac muscle cells.

Whether NRG-mediated cardiac muscle cell differentiation opposes proliferation by other growth factors was assessed by mixing rhNRGβ2 with IGF-I in a cardiac myocyte DNA synthesis assay. This issue could be of significance since *in vivo* myocardial cells expose to multiple peptide hormones and growth factors. In this experiment, an optimal concentration of IGF-I (10^{-9}M) was combined with either 10^{-10}M or 10^{-8}M NRG. As shown in Figure 4, unlike NRG alone, the lower concentration of NRG had a small negative effect on the DNA synthesis, whereas the higher concentration significantly blocked the response of myocardial cells to IGF-I stimulation.

The latter observation supported the above data that a specific intracellular pathway was activated by the higher concentration of NRG.

Phenotype of cultured neonatal rat cardiac muscle cells was evaluated by immunofluorescent staining with antibody specific to sarcomere protein, α-actinin, and by Phalloidin staining for actin fibers. Cells cultured in serum-free medium showed disorganisation of cytoskeleton structures (Figure 5), whereas cells cultured with NRG had much organised actin fibers (Figure 5). Myocardial cell phenotype was further evaluated by examining the sarcomeric structures. As shown in Figure 6, in cells with the immunofluorescent staining with anti-α-actinin antibody, NRG dramatically improved the assembly of sarcomeres in cultured cells, as compared with cells without NRG adminstration. The ligand concentration also play a role in stimulation, as the higher concentration of NRG (10^{-8}M) results in more organised sarcomeres (Figure 6). Cell-cell adhesions were also enhanced by NRG stimulation particularly for cells stimulated by the higher concentration of the ligand. Cell structures of NRG treated cells were also compared with IGF-1 or PE stimulated cells. IGF-1 has no clear effect on cell sarcomeres and cell-cell adhesions (Figure 7), whereas, PE stimulation resulted two type of cells: type A is similar to NRG stimulated cells, which is well differentiated with very organised sarcomeric structures; type B has a large cell surface area and relatively poor sarcomeric structures and cell-cell adhesions (Figure 8). Importantly, when cells were stimulated with NRG (10^{-8}M) and IGF-1 together, they showed improved sacomere structures comparing cells stimulated with IGF-1 alone (Figure 7). When NRG (10^{-8}M) and PE were used together in stimulation of cells, only type A cells were observed (Figure 8). Cardiac muscle cell differentiation and maturation were accompanied with increased expression of MHC-α and decreased expression of MHC-β. PE as a putative hypertrophy factor suppresses the MHC-α expression (Figure 9). However, NRG alone had a clear positive effect on MHC-α expression and was able to override the PE-mediated negative effect on MHC-α expression (Figure 9).

DISCUSSION

Evidence provided indicates that ligand (NRG) concentration is a an important factor in determining either the transient or sustained activation states of MAP kinases. The latter results in increased expression level of the Cdk inhibitor, $p21^{CIP1}$, and is associated with decreased DNA synthesis in embryonic myocardial cells. This finding provides clear support that the ligand gradient may decide cell fate in cell differentiation and embryo development, and further furnishes molecular insights on how intracellular signalling pathways distinguish the signal strength based on ligand concentrations.

The importance of ligand concentration in cell differentiation has been suspected for some time based on the following observations:
i) embryo developmental patterning is associated with a ligand gradient;
ii) ligand concentration is critical for cell differentiation *in vitro*, and
iii) overexpression of receptors in cells changes their fate in response to ligand stimulation.

Taking these observations into consideration, NRG concentration-dependent MAP kinase activation in embryonic myocardial cells establishes a model for further delineating the mechanisms of erbB receptor-coupled cell signalling in reaction to changes in ligand concentration.

The notion that NRG is a myocardial cell differentiation factor is supported by the finding that NRG induces expression of $p21^{CIP1}$ in embryonic myocardial cells. As $p21^{CIP1}$ is well documented to be an inhibitor of Cdk, which promotes entry from the G1 to the S phase of the cell cycle, increased expression of this protein in myocardial cells could be critical for the initiation of terminal differentiation. This is also supported by previous findings that $p21^{CIP1}$ expression increases *in vivo* with the onset of myocardial cell terminal differentiation (Parker et al. (1995) Science 267:1024-1027), as well as with skeletal muscle cell differentiation (Dias et al. (1994) Semin. Diagn Pathol. 11:3-14). In the latter process, increased $p21^{CIP1}$ expression eventually results in an exit from the cell cycle and differentiation. Since increase in $p21^{CIP1}$ expression occurs prior to that of other cell cycle regulators, it is used as an early marker for skeletal muscle differentiation. As demonstrated here, expression of $p21^{CIP1}$ is concomitant with the decrease in DNA synthesis in NRG-stimulated myocardial cells, suggesting the physiological role of NRG-stimulated $p21^{CIP1}$ expression in

these cells. Furthermore, the inhibition of both MAP kinases and $p21^{CIP1}$ by the ERK kinases inhibitor assessed that NRG-stimulated $p21^{CIP1}$ expression is a direct result of activation of MAP kinases.

The sustained activation of MAP kinases is required for induction of $p21^{CIP1}$ constitutive expression in cultured myocardial cells, whereas transient MAP kinase activation results in temporal expression of $p21^{CIP1}$. The latter is presumably insufficient to regulate the Cdk activity, since $p21^{CIP1}$ will be quickly degraded and constitutive expression is essential for blocking the cyclin/Cdk complex. In PC12 cells, sustained activation of the MAP kinase pathway is confined to a response to specific signals from NGF receptors. The sustained activation of MAP kinases causes PC12 cell differentiation becoming neuronal cells. This pathway in cardiac myocytes, however, is able to differentially respond to NRG concentration-based signal strength.

Further evidences support that NRG is a differentiation factor are that NRG stimulate assembly of sarcomeric and cytoskeleton structures, which occur as myocardial progenitor cells differentiate to cardiac muscle cells. Previous observation also indicated that more differentiated cells have more organised sarcomeres (Rumynatsev, P.P. (1977) in International Review Cytology 51, pp 187-273). In a comparison of cells stimulated with either PE or IGF-1, NRG-stimulated cells have the best organised sarcomeres. More importantly, when NRG is mixed with PE or IGF-1, NRG greatly improved sarcomeres, indicating that NRG is dominant in stimulation of sarcomere assembly in presence of other cell signals. NRG overrides the PE-mediated negative regulation of MHC-α expression, indicating that NRG is involved in the maintenance of adult type of contractile proteins. As previous studies indicated that NRG, ErbB2 and ErbB4 are expressed in adult heart, NRG should play a role in the maintenance of myocardial cell differentiation state.

Two very important features of heart failure associated with cardiomyopathy in patients are disarrays of myofibers and sarcomeres. The former is the loose of the cell-cell adhesion and the latter is the loose of the sarcomere organisation. These pathological conditions widely exist from congestive heart failure to dilated cardiomyopathy and severely affect heart function. Currently no treatment is target on the assembly of cell-cell adhesion and sacomere structures. NRG clearly plays a role in the process of the assembly and maintenance of cell-cell adhesion and sarcomeric

structures. That NRG stimulates myocardial cell differentiation and the assembly of sarcomeric structures indicates that cardiac muscle cell differentiation is associated with its cell structure remodelling. Such a conclusion is consistent with general observation from heart muscle cell differentiation during heart development: differentiated muscle cells always contain well organised sarcomeres.

In summary, that NRG is a differentiation factor for myocardial cells is supported by following evidence:

i) NRG stimulates sustained activation of MAP kinases;

ii) NRG enhances $p21^{CIP1}$ expression;

ii) NRG inhibits IGF-1-stimulated DNA synthesis; and

iv) NRG stimulates the myocardial cell assembly of sarcomeric and cytoskeleton structures.

v) NRG stimulates expression of the adult-type MHC gene.

THERAPEUTIC USE

The present invention provides methods for treating or preventing heart failure or cardiac muscle cell hypertrophy in a mammal by providing an effective amount of a neuregulin. Preferably, the mammal is a human patient suffering from or at risk of developing heart failure.

The present invention is useful in preventing heart failure and cardiomyopathy in patients being treated with a drug which cause cardiac hypertrophy or congestive heart failure, e.g., fludrocortisone acetate or herceptin. In the method of the invention, a neuregulin polypeptide can be given prior to, simultaneously with, or subsequent to a drug which causes cardiac diseases.

In the therapeutic method of the invention, a neuregulin polypeptide is administered to a human patient chronically or acutely, for example by injection into the patient's vein. Optionally, neuregulin is administered chronically in combination with an effective amount of a compound that acts to suppress a different hypertrophy induction pathway than a neuregulin Additional optional components include a cardiotrophic inhibitor such as a Ct-1 antagonist, an ACE inhibitor, such as captopril, and/or human growth hormone and/or IGF-I in the case of congestive heart failure, or with another anti-hypertrophic, myocardiotrophic factor, anti-arrhythmic, or inotropic factor in the case of other types of heart failure or cardiac disorder.

The present invention can be combined with current therapeutic approaches for treatment of heart failure, e.g., with ACE inhibitor treatment. ACE inhibitors are angiotensin-converting enzyme inhibiting drugs which prevent the conversion of angiotensin I to angiotensin II. The ACE inhibitors may be beneficial in congestive heart failure by reducing systemic vascular resistance and relieving circulatory congestion. ACE inhibitors include drugs designated by the trademarks Accupril® (quinapril), Altace® (ramipril), Capoten® (captopril), Lotensin® (benazepril), Monopril® (fosinopril), Prinivil® (lisinopril), Vasotec® (enalapril), and Zestril® (lisinopril).

The present invention can be combined with the administration of drug therapies for the treatment of heart diseases such as hypertension. For example, a neuregulin polypeptide can be administered with endothelin receptor antagonists, for example, and antibody to the endothelin receptor, and peptide or other such small molecule antagonists; β-adrenoreceptor antagonists such as carvedilol; α_1-adrenoreceptor antagonists; anti-oxidants; compounds having multiple activities (e.g., β-blocker/α-blocker/anti-oxidant); carvedilol-like compounds or combinations of compounds providing multiple functions found in carvedilol; growth hormone, etc.

Neuregulin agonists alone or in combination with other hypertrophy suppressor pathway agonists or with molecules that antagonise known hypertrophy induction pathways, are useful as drugs for *in vivo* treatment of mammals experiencing heart failure, so as to prevent or lessen heart failure effects.

Therapeutic formulations of agonist(s) for treating heart disorders are prepared for storage by mixing the agonist(s) having the desired degree of purity with optional physiologically acceptable carriers, excipients, or stabilisers (Remington's Pharmaceutical Sciences, 16th edition, Oslo, A., Ed., 1980), in the form of lyophilised cake or aqueous solutions. Acceptable carriers, excipients, or stabilisers are non-toxic to recipients at the dosages and concentrations employed, and include buffers such as phosphate, citrate, and other organic acids; antioxidants including ascorbic acid; low molecular weight (less than about 10 residues) polypeptides; proteins, such as serum albumin, gelatin, or immunoglobins; hydrophilic polymers such as polyvinylpyrrolidone; amino acids such as glycine, glutamine, asparagine, arginine or lysine; monosaccharides, disaccharides, and other carbohydrates including glucose, mannose, or dextrins; chelating agents such as EDTA;

sugar alcohols such as mannitol or sorbitol; salt-forming counter ions such as sodium; and/or non-ionic surfactants such as Tween, Pluronics, or polyethylene glycol (PEG). The antagonist(s) are also suitably linked to one of a variety of nonproteinaceous polymers, e.g., polyethylene glycol, polypropylene glycol, or polyalkylenes, in the manner set forth in US Patent Nos. 4,640,835; 4,496,689; 4,301,144; 4,670,417; 4,791,192 or 4,179,337. The amount of carrier used in a formulation may range from about 1 to 99%, preferably from about 80 to 99%, optimally between 90 and 99% by weight.

The agonist(s) to be used for *in vivo* administration should be sterile. This is readily accomplished by methods known in the art, for example, by filtration through sterile filtration membranes, prior to or following lyophilisation and reconstitution. The agonist(s) ordinarily will be stored in lyophilised form or in solution.

Therapeutic agonist compositions generally are placed into a container having a sterile access port, for example, an intravenous solution bag or vial having a stopper pierceable by a hypodermic injection needle. The agonist(s) administration is in a chronic fashion only, for example, one of the following routes: injection or infusion by intravenous, intraperitoneal, intracerebral, intramuscular, intraocular, intraarterial, or intralesional routes, orally or using sustained-release systems as noted below. Agonist(s) are administered continuously by infusion or by periodic bolus injection if the clearance rate is sufficiently slow, or by administration into the blood stream or lymph. The preferred administration mode is targeted to the heart, so as to direct the molecule to the source and minimise side-effects of the agonists.

Suitable examples of sustained-release preparations include semipermeable matrices of solid hydrophobic polymers containing the protein, which matrices are in form of shaped articles, e.g., films, or microcapsules. Examples of sustained-release matrices include polyesters, hydrogels (e.g., poly(2-hydroxyethyl-methacrylate) as described by Langer et al. (1981) J. Biomed. Mater. Res. 15: 167-277 and Langer (1982) Chem. Tech. 12: 98-105, or poly(vinyl alcohol)), polylactides (US Patent No 3,773,919, EP 58,481), copolymers of L-glutamic acid and gamma ethyl-L-glutamate (Sidman et al. (1983) Biopolymers 22: 547-556), non-degradable ethylene-vinyl acetate (Langer et al. (1981) supra) degradable lactic acid-glycolic acid copolymers such as the Lupron Depot™ (injectable

microspheres composed of lactic acid-glycolic acid copolymer and leuprolide acetate), and poly-D-(-)-3-hydroxybutyric acid (EP 133,988).

The agonist(s) also may be entrapped in microcapsules prepared, for example, by coacervation techniques or by interfacial polymerisation (for example, hydroxymethylcellulose or gelatin-microcapsules and poly-[methylmethacylate] microcapsules, respectively, in colloidal drug delivery systems (for example, liposomes, albumin microspheres, microemulsions, nano-particles and nanocapsules), or in macroemulsions. Such techniques are disclosed in Remington's Pharmaceutical Sciences, *supra*.

While polymers such as ethylene-vinyl acetate and lactic acid-glycolic acid enable release of molecules for over 100 days, certain hydrogels release molecules for shorter time periods. When encapsulated molecules remain in the body for a long time, they may denature or aggregate as a result of exposure to moisture at 37°C, resulting in a loss of biological activity and possible changes in immunogenicity. Rational strategies can be devised for stabilisation depending on the mechanism involved, e.g., using appropriate additives, and developing specific polymer matrix compositions.

Sustained-release agonist(s) compositions also include liposomally entrapped agonists(s). Liposomes containing agonists(s) are prepared by methods known *per se*: DE 3,218,121; Epstein et al. (1985) Proc. Natl. Acad. Sci. USA 82: 3688-3692; Hwang et al. (1980) Proc. Natl. Acad. Sci. USA 77: 4030-4034; EP 52,322; EP 36,676; EP 88,046; EP 143,949; EP 142,641; Japanese patent application 83-118008; US Patent Nos. 4,485,045 and 4,544,545; and EP 102, 324. Ordinarily the liposomes are of the small (about 200-800 Å) unilamellar type in which the lipid content is greater than about 30 mol% cholesterol, the selected proportion being adjusted for the optimal agonist therapy. A specific example of suitable sustained-release formulation is in EP 647,449.

An effective amount of NRG to be employed therapeutically will depend, for example, upon the therapeutic objectives, the route of administration, and the condition of the patient. Accordingly, it will usually be necessary for the clinician to titre the dosage and modify the route of administration as required to obtain the optimal therapeutic effect.

NRG optionally is combined with or administered in concert with other agents for treating congestive heart failure, including ACE inhibitors, CT-1 inhibitors, human growth hormone, and/or IGF-I. The effective

amounts of such agents, if employed, will be at the clinician's discretion. Dosage administration and adjustment are determined by methods known to those skilled in the art to achieve the best management of congestive heart failure and ideally takes into account use of diuretics or digitalis, and conditions such as hypotension and renal impairment. The dose will additionally depend on such factors as the type of drug used and the specific patient being treated. Typically the amount employed will be the same dose as that used if the drug were to be administered without agonist; however, lower doses may be employed depending on such factors as the presence of side-effects, the condition being treated, the type of patient, and the type of agonists and drug, provided the total amount of agents provides an effective dose for the condition being treated.

Thus, for example, in the case of ACE inhibitors, a test dose of enalapril is 5 mg, which is then increased up to 10-20 mg per day, once a day, as the patient tolerates it. As another example, captopril is initially administered orally to human patients in a test dose of 6.25 mg and the dose is then escalated, as the patient tolerates it to 25 mg twice per day (BID) or three times per day (TID) and may be titrated to 50 mg BID or TID. Tolerance level is estimated by determining whether decrease in blood pressure is accompanied by signs of hypotension. If indicated, the dose may be increased up to 100 mg BID or TID. Captopril is produced for administration as the active ingredient, in combination with hydrochlorothiazide, and as a pH stabilised core having an enteric or delayed release coating which protects captopril until it reaches the colon. Captopril is available for administration in tablet or capsule form. A discussion of the dosage. Administration, indications and contraindications associated with captopril and other ACE inhibitors can be found in the Physicians Desk Reference, Medical Economics Data Production Co., Montvale, NJ. 2314-2320 (1994).

In an example of an injectable therapeutic composition of neuregulin, the formulation contains 1% neuregulin and 99% saline, where neuregulin is a polypeptide thereof. In another example of an injectable therapeutic composition of neuregulin, the formulation contains 5% of the neuregulin polypeptide, 1% ACE inhibitor captopril, and 94% saline.

It will be appreciated by persons skilled in the art that numerous variations and/or modifications may be made to the invention as shown in the specific embodiments without departing from the spirit or scope of the invention as broadly described. The present embodiments are, therefore, to be considered in all respects as illustrative and not restrictive.

Dated this 21st day of December 1998

THE VICTOR CHANG CARDIAC
RESEARCH INSTITUTE
Patent Attorneys for the Applicant:

F B RICE & CO

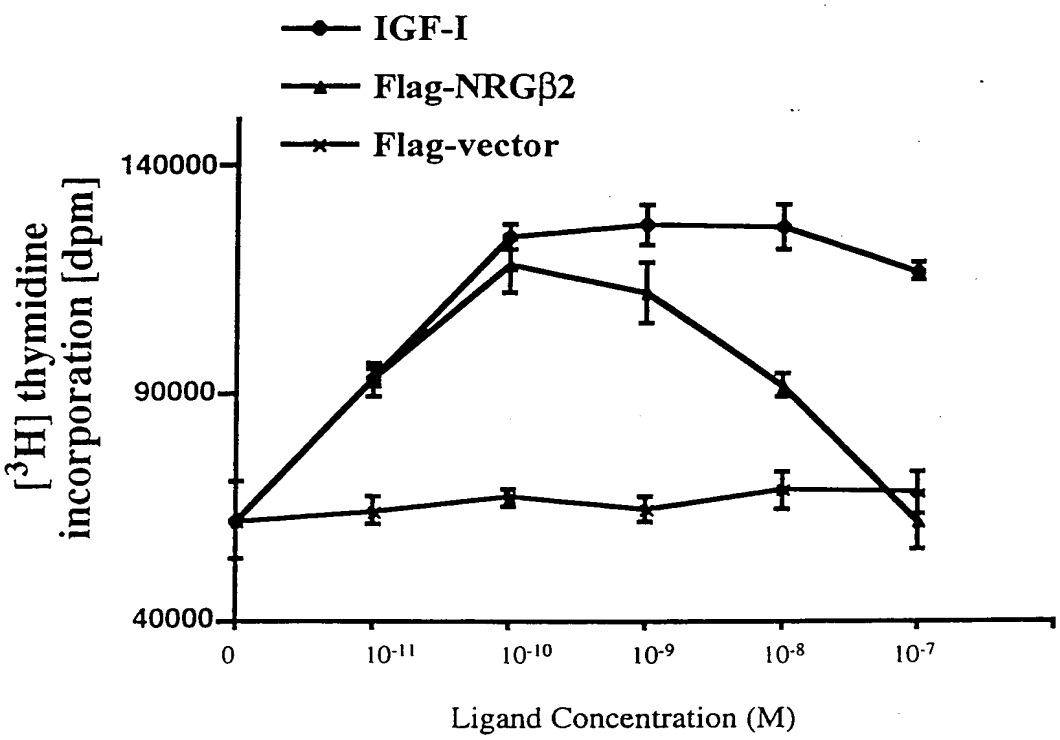
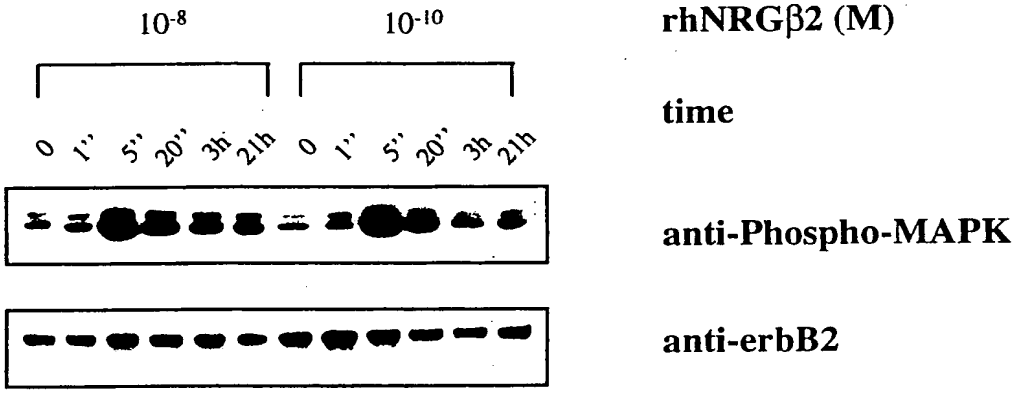

IGF-I
Flag-NRGβ2
Flag-vector
[3H] thymidine incorporation [dpm]
140000
90000
40000
0
10-11
10-10
10-9
10-8
10-7
Ligand Concentration (M)

Figure 1

a


10-8
10-10
rhNRGβ2 (M)
0 1'' 5'' 20'' 3h 21h 0 1'' 5'' 20'' 3h 21h
time
anti-Phospho-MAPK
anti-erbB2

b

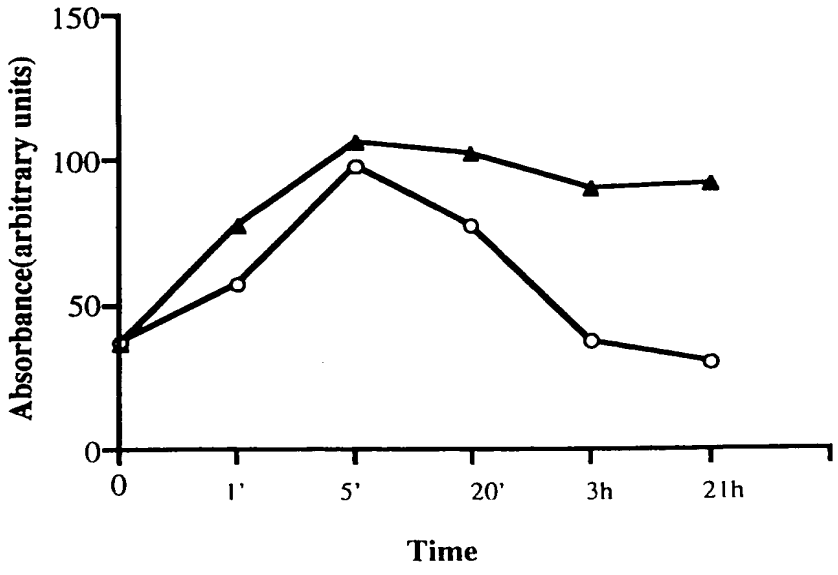

150
100
50
0
Absorbance(arbitrary units)
0
1'
5'
20'
3h
21h
Time

Figure 2

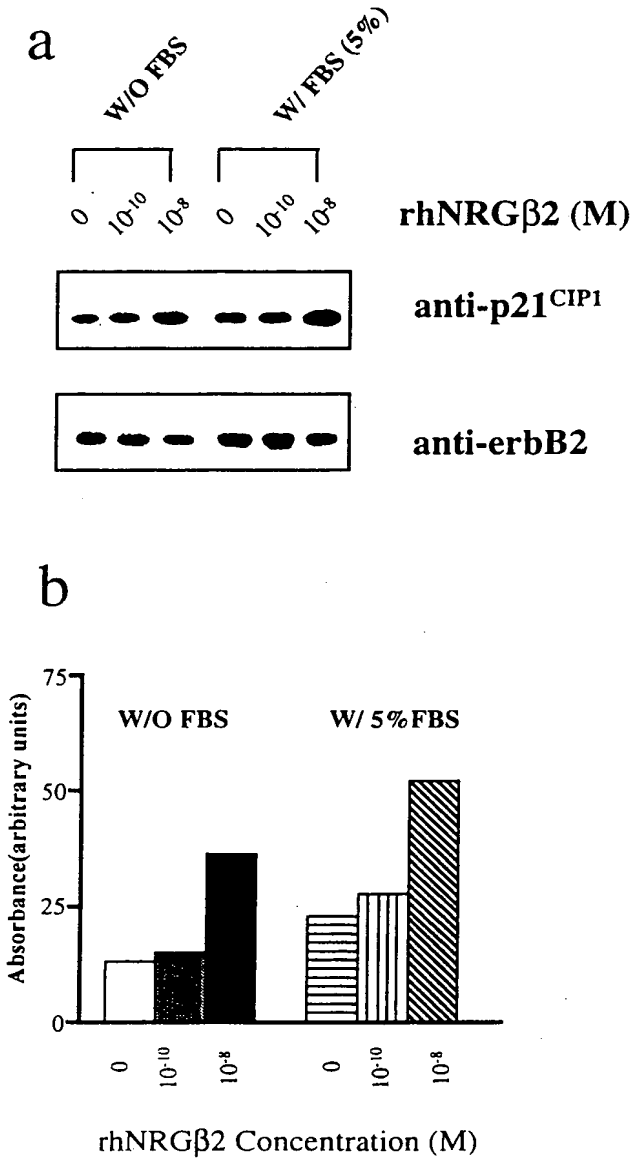

a
W/O FBS
W/ FBS (5%)
0
10-10
10-8
0
10-10
10-8
rhNRGβ2 (M)
anti-p21CIP1
anti-erbB2
b
75
50
25
0
Absorbance(arbitrary units)
W/O FBS
W/ 5%FBS
0
10-10
10-8
0
10-10
10-8
rhNRGβ2 Concentration (M)

Figure 3

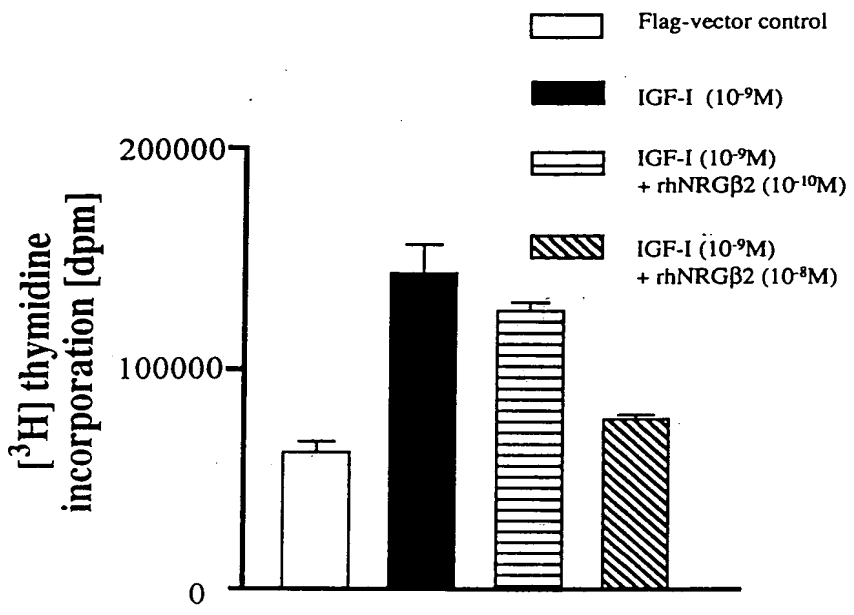

Flag-vector control
IGF-I (10-9M)
IGF-I (10-9M)
+ rhNRGβ2 (10-10M)
IGF-I (10-9M)
+ rhNRGβ2 (10-8M)
200000
100000
0
[3H] thymidine
incorporation [dpm]

Figure 4

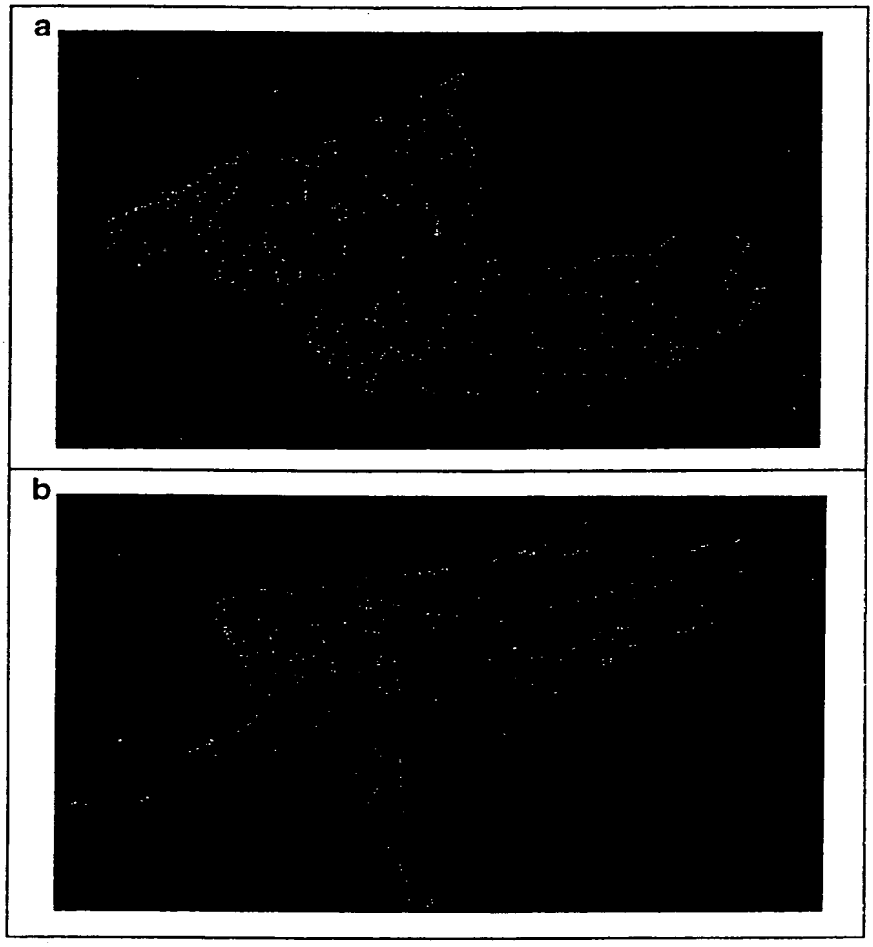


Figure 5

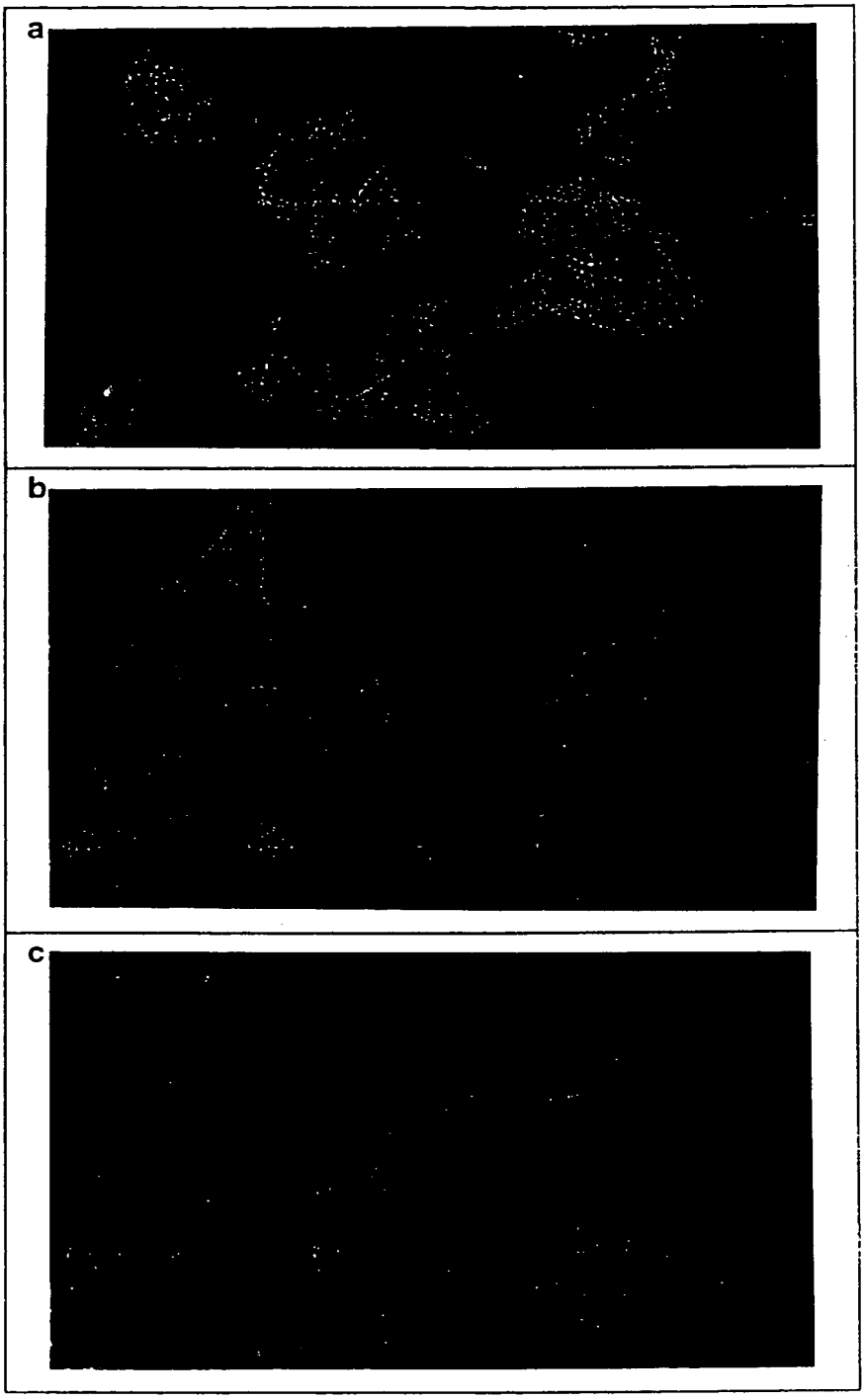


Figure 6

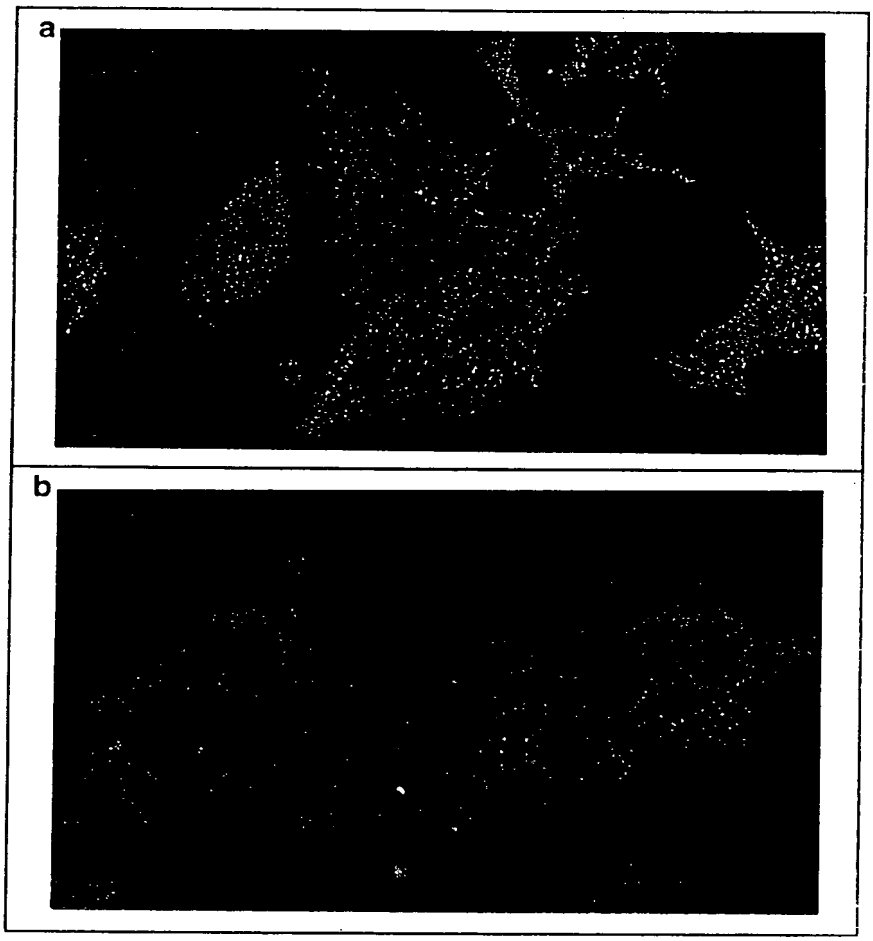


Figure 7

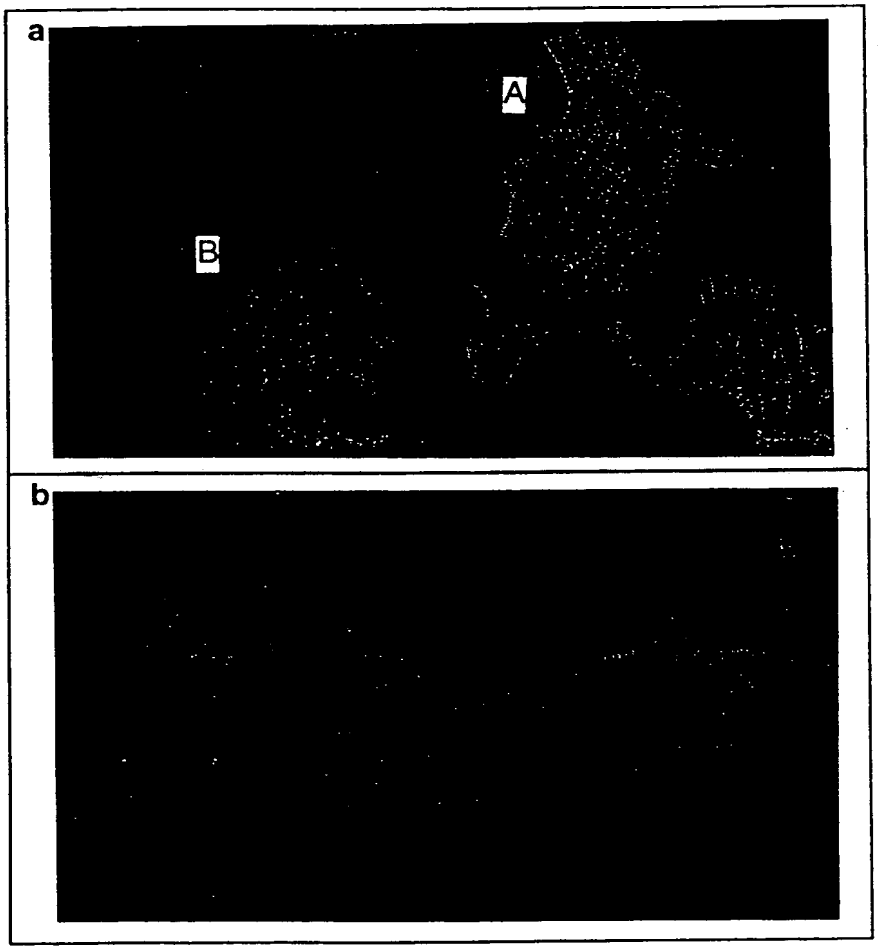


Figure 8

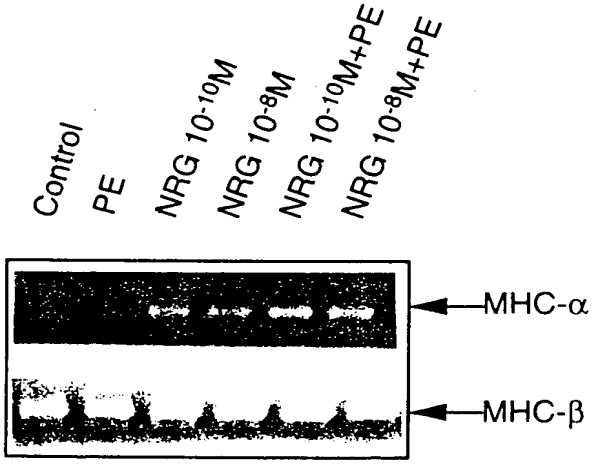

Control
PE
NRG 10-10M
NRG 10-8M
NRG 10-10M+PE
NRG 10-8M+PE
MHC-α
MHC-β

Figure 9